

## NUCLEOTIDE SEQUENCES WHICH CODE FOR THE *dead* GENE

### [0001] BACKGROUND OF THE INVENTION

The invention provides nucleotide sequences from coryneform bacteria which code for the *dead* gene and a process for the fermentative preparation of amino acids using bacteria in which the *dead* gene is attenuated. All references cited herein are expressly incorporated by reference. Incorporation by reference is also designated by the term "I.B.R." following any citation.

[0002] L-Amino acids, in particular L-lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

[0003] It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

[0004] Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce amino acids are obtained in this manner.

[0005] Methods of the recombinant DNA technique have also been employed for some years for improving the strain of

Corynebacterium strains which produce L-amino acid, by amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

[0006] The invention provides new measures for improved fermentative preparation of amino acids.

#### [0007] BRIEF SUMMARY OF THE INVENTION

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Lysine is particularly preferred.

[0008] When L-lysine or lysine are mentioned in the following, not only the bases but also the salts, such as e.g. lysine monohydrochloride or lysine sulfate, are meant by this.

[0009] The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the *dead* gene, chosen from the group consisting of

- a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide which is complementary to the polynucleotides of a) or b), and

- d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of DNA/RNA helicase.

[0010] The invention also provides the above-mentioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

- (i) the nucleotide sequence, shown in SEQ ID No.1, or
- (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or
- (iii) at least one sequence which hybridizes with the sequences complementary to sequences (i) or (ii), and optionally
- (iv) sense mutations of neutral function in (i).

[0011] The invention also provides:

a polynucleotide, in particular DNA, which is capable of replication and comprises the nucleotide sequence as shown in SEQ ID No.1;

a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing parts of the polynucleotide according to the invention, but at least 15 successive nucleotides of the sequence claimed,

and coryneform bacteria in which the deadD gene is attenuated, in particular by an insertion or deletion.

[0012] The invention also provides polynucleotides, which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide sequence mentioned.

[0013] BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Map of the plasmid pXK99E,

Figure 2: Map of the plasmid pXK99EdeaD.

[0014] The abbreviations and designations used have the following meaning.

|         |   |
|---------|---|
| Kan:    | Kanamycin resistance gene aph(3')-IIa from Escherichia coli |
| BstEII  | Cleavage site of the restriction enzyme BstEII              |
| HindIII | Cleavage site of the restriction enzyme HindIII             |
| NcoI    | Cleavage site of the restriction enzyme NcoI                |
| XbaI    | Cleavage site of the restriction enzyme XbaI                |
| Ptrc    | trc promoter  |
| T1      | Termination region T1                                       |
| T2      | Termination region T2                                       |

LacIq            LacIq repressor of the lac operon of  
                 Escherichia coli

OriV            Replication origin ColE1 from E. coli

DeaD            Cloned region of the deaD gene

**[0015] DETAILED DESCRIPTION OF THE INVENTION**

Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for DNA/RNA helicase or to isolate those nucleic acids or polynucleotides or genes which have a high similarity with the sequence of the deaD gene. They can also be attached as a probe to so-called "arrays", "micro arrays" or "DNA chips" in order to detect and to determine the corresponding polynucleotides or sequences derived therefrom, such as e.g. RNA or cDNA.

**[0016]** Polynucleotides which comprise the sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for DNA/RNA helicase can be prepared by the polymerase chain reaction (PCR).

**[0017]** Such oligonucleotides which serve as probes or primers comprise at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24, very particularly preferably at least 15, 16, 17, 18 or 19 successive nucleotides. Oligonucleotides with a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 or at least 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100,

150, 200, 250 or 300 nucleotides are optionally also suitable.

[0018] "Isolated" means separated out of its natural environment.

[0019] "Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

[0020] The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90% and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

[0021] "Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

[0022] The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of DNA/RNA helicase and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

[0023] The invention furthermore relates to a process for the fermentative preparation of amino acids chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine using coryneform bacteria which in particular

already produce amino acids and in which the nucleotide sequences which code for the *dead* gene are attenuated, in particular eliminated or expressed at a low level.

[0024] The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

[0025] By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

[0026] The microorganisms provided by the present invention can prepare amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among experts for its ability to produce L-amino acids.

[0027] Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains

*Corynebacterium glutamicum* ATCC13032

*Corynebacterium acetoglutamicum* ATCC15806

*Corynebacterium acetoacidophilum* ATCC13870

*Corynebacterium melassecola* ATCC17965

*Corynebacterium thermoaminogenes* FERM BP-1539

Brevibacterium flavum ATCC14067

Brevibacterium lactofermentum ATCC13869 and

Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains prepared therefrom.

[0028] The new *deaD* gene from *C. glutamicum* which codes for the enzyme DNA/RNA helicase has been isolated.

[0029] To isolate the *deaD* gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first set up in *Escherichia coli* (*E. coli*). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: *Gene und Klone, Eine Einführung in die Gentechnologie* (Verlag Chemie, Weinheim, Germany, 1990) *I.B.R.*, or the handbook by Sambrook et al.: *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1989) *I.B.R.* may be mentioned as an example. A well-known gene library is that of the *E. coli* K-12 strain W3110 set up in  $\lambda$  vectors by Kohara et al. (*Cell* 50, 495 -508 (1987)) *I.B.R.* Bathe et al. (*Molecular and General Genetics*, 252:255-265, 1996) *I.B.R.* describe a gene library of *C. glutamicum* ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, *Proceedings of the National Academy of Sciences USA*, 84:2160-2164 *I.B.R.*) in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, *Nucleic Acids Research* 16:1563-1575 *I.B.R.*).

[0030] Börmann et al. (*Molecular Microbiology* 6(3), 317-326) (1992) *I.B.R.* in turn describe a gene library of *C. glutamicum* ATCC13032 using the cosmid pHC79 (Hohn and Collins, 1980, *Gene* 11, 291-298 *I.B.R.*).

[0031] To prepare a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322 (Bolivar, 1979, *Life Sciences*, 25, 807-818 *I.B.R.*) or pUC9 (Vieira et al., 1982, *Gene*, 19:259-268 *I.B.R.*). Suitable



hosts are, in particular, those *E. coli* strains which are restriction- and recombination-defective, such as, for example, the strain DH5 $\alpha$ mc<sup>r</sup>, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) *I.B.R.* The long DNA fragments cloned with the aid of cosmids or other  $\lambda$  vectors can then in turn be subcloned and subsequently sequenced in the usual vectors which are suitable for DNA sequencing, such as is described e. g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977) *I.B.R.*

[0032] The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)) *I.B.R.*, that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) *I.B.R.* or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)) *I.B.R.*

[0033] The new DNA sequence of *C. glutamicum* which codes for the *deaD* gene and which, as SEQ ID No. 1, is a constituent of the present invention has been found. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the *deaD* gene product is shown in SEQ ID No. 2.

[0034] Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. It is furthermore known that changes on the N and/or C terminus

of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)) *I.B.R.*, in O'Regan et al. (Gene 77:237-251 (1989)) *I.B.R.*, in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)) *I.B.R.*, in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) *I.B.R.* and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

[0035] In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

[0036] Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) *I.B.R.* and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)) *I.B.R.* The hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i. e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996 *I.B.R.*).





mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair (bp) in a gene lead to frame shift mutations, as a consequence of which incorrect amino acids are incorporated or translation is interrupted prematurely. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art and can be found in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) *I.B.R.*, that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990 *I.B.R.*) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986) *I.B.R.*

**[0044]** A common method of mutating genes of *C. glutamicum* is the method of "gene disruption" and "gene replacement" described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)) *I.B.R.*

**[0045]** In the method of gene disruption a central part of the coding region of the gene of interest is cloned in a plasmid vector which can replicate in a host (typically *E. coli*), but not in *C. glutamicum*. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)) *I.B.R.*, pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994) *I.B.R.*), pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992) *I.B.R.*), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84 *I.B.R.*; US Patent 5,487,993 *I.B.R.*), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993) *I.B.R.*) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516 *I.B.R.*). The plasmid vector which contains the central part of the coding region of the gene is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is

described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)) *I.B.R.* Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)) *I.B.R.*, Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) *I.B.R.* and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)) *I.B.R.* After homologous recombination by means of a "cross-over" event, the coding region of the gene in question is interrupted by the vector sequence and two incomplete alleles are obtained, one lacking the 3' end and one lacking the 5' end. This method has been used, for example, by Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580 (1994)) *I.B.R.* to eliminate the *recA* gene of *C. glutamicum*.

[0046] In the method of "gene replacement", a mutation, such as e.g. a deletion, insertion or base exchange, is established in vitro in the gene of interest. The allele prepared is in turn cloned in a vector which is not replicative for *C. glutamicum* and this is then transferred into the desired host of *C. glutamicum* by transformation or conjugation. After homologous recombination by means of a first "cross-over" event which effects integration and a suitable second "cross-over" event which effects excision in the target gene or in the target sequence, the incorporation of the mutation or of the allele is achieved. This method was used, for example, by Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998)) *I.B.R.* to eliminate the *pyc* gene of *C. glutamicum* by a deletion.

[0047] A deletion, insertion or a base exchange can be incorporated into the *dead* gene in this manner.

[0048] In addition, it may be advantageous for the production of L-amino acids to enhance, in particular over-express, one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid

cycle, of the pentose phosphate cycle, of amino acid export and optionally regulatory proteins, in addition to the attenuation of the *dead* gene.

[0049] The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene or allele which codes for a corresponding enzyme (protein) having a high activity, and optionally combining these measures.

[0050] By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

[0051] Thus, for the preparation of L-amino acids, in addition to the attenuation of the *dead* gene at the same time one or more of the genes chosen from the group consisting of

- the *dapA* gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335 *I.B.R.*),
- the *gap* gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), *Journal of Bacteriology* 174:6076-6086 *I.B.R.*),
- the *tpi* gene which codes for triose phosphate isomerase (Eikmanns (1992), *Journal of Bacteriology* 174:6076-6086 *I.B.R.*),

- the *pgk* gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086 *I.B.R.*),
- the *zwf* gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661 *I.B.R.*),
- the *pyc* gene which codes for pyruvate carboxylase (DE-A-198 31 609 *I.B.R.*),
- the *mgo* gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998) *I.B.R.*),
- the *lysC* gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512; EP-B-0387527 *I.B.R.*; EP-A-0699759 *I.B.R.*),
- the *lysE* gene which codes for lysine export (DE-A-195 48 222 *I.B.R.*),
- the *hom* gene which codes for homoserine dehydrogenase (EP-A 0131171 *I.B.R.*),
- the *ilvA* gene which codes for threonine dehydratase (Möckel et al., Journal of Bacteriology (1992) 8065-8072 *I.B.R.*) or the *ilvA(Fbr)* allele which codes for a "feed back resistant" threonine dehydratase (Möckel et al., (1994) Molecular Microbiology 13: 833-842 *I.B.R.*),
- the *ilvBN* gene which codes for acetohydroxy-acid synthase (EP-B 0356739 *I.B.R.*),
- the *ilvD* gene which codes for dihydroxy-acid dehydratase (Sahm and Eggeling (1999) Applied and Environmental Microbiology 65: 1973-1979 *I.B.R.*),
- the *zwa1* gene which codes for the *Zwa1* protein (DE: 19959328.0 *I.B.R.*, DSM 13115)

can be enhanced, in particular over-expressed.



[0052] It may furthermore be advantageous for the production of amino acids, in addition to the attenuation of the *deaD* gene, at the same time for one or more of the genes chosen from the group consisting of

- the *pck* gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1 *I.B.R.*, DSM 13047),
- the *pgi* gene which codes for glucose 6-phosphate isomerase (US 09/396,478 *I.B.R.*, DSM 12969),
- the *poxB* gene which codes for pyruvate oxidase (DE:1995 1975.7 *I.B.R.*, DSM 13114),
- the *zwa2* gene which codes for the Zwa2 protein (DE: 19959327.2 *I.B.R.*, DSM 13113)

to be attenuated, in particular for the expression thereof to be reduced.

[0053] In addition to the attenuation of the *deaD* gene it may furthermore be advantageous for the production of amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982 *I.B.R.*).

[0054] The invention also provides the microorganisms prepared according to the invention, and these can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of L-amino acids. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991) *I.B.R.*) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)) *I.B.R.*

[0055] The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981) I.B.R.

[0056] Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as, for example, soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

[0057] Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

[0058] Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.



09063790-092734  
492260-064960

[0064] The isolation of plasmid DNA from *Escherichia coli* and all techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA) *I.B.R.* Methods for transformation of *Escherichia coli* are also described in this handbook.

[0065] The composition of the usual nutrient media, such as LB or TY medium, can also be found in the handbook by Sambrook et al.

[0066] Example 1

Preparation of a genomic cosmid gene library from *C. glutamicum* ATCC 13032

[0067] Chromosomal DNA from *C. glutamicum* ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) *I.B.R.* and partly cleaved with the restriction enzyme *Sau3AI* (Amersham Pharmacia, Freiburg, Germany, Product Description *Sau3AI*, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description *SAP*, Code no. 1758250 *I.B.R.*). The DNA of the cosmid vector *SuperCos1* (Wahl et al. (1987), Proceedings of the National Academy of Sciences, USA 84:2160-2164 *I.B.R.*), obtained from Stratagene (La Jolla, USA, Product Description *SuperCos1* Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme *XbaI* (Amersham Pharmacia, Freiburg, Germany, Product Description *XbaI*, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

[0068] The cosmid DNA was then cleaved with the restriction enzyme *BamHI* (Amersham Pharmacia, Freiburg, Germany, Product Description *BamHI*, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase

(Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217 I.B.R.).

[0069] For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575 I.B.R.) the cells were taken up in 10 mM MgSO<sub>4</sub> and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor) I.B.R., the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190 I.B.R.) + 100 µg/ml ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

#### [0070] Example 2

Isolation and sequencing of the deaD gene

[0071] The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

[0072] The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-

01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor) *I.B.R.*, the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol. Letters, 123:343-7 *I.B.R.*) into the *E. coli* strain DH5 $\alpha$ mcr (Grant, 1990, Proceedings of the National Academy of Sciences, U.S.A., 87:4645-4649 *I.B.R.*). Letters, 123:343-7 *I.B.R.*) and plated out on LB agar (Lennox, 1955, Virology, 1:190 *I.B.R.*) with 50  $\mu$ g/ml zeocin.

[0073] The plasmid preparation of the recombinant clones was carried out with the Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academies of Sciences, U.S.A., 74:5463-5467 *I.B.R.*) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067) *I.B.R.* The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

[0074] The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231 *I.B.R.*) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig. The computer-assisted coding region analyses were prepared with the XNIP program (Staden, 1986,

Nucleic Acids Research, 14:217-231 *I.B.R.*). Further analyses can be carried out with the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402 *I.B.R.*) against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA) *I.B.R.*

[0075] The relative degree of substitution or mutation in the polynucleotide or amino acid sequence to produce a desired percentage of sequence identity can be established or determined by well-known methods of sequence analysis. These methods are disclosed and demonstrated in Bishop, et al. "DNA & Protein Sequence Analysis (A Practical Approach)", Oxford Univ. Press, Inc. (1997) *I.B.R.* and by Steinberg, Michael "Protein Structure Prediction" (A Practical Approach), Oxford Univ. Press, Inc. (1997) *I.B.R.*

[0076] The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence showed an open reading frame of 1875 bp, which was called the *deaD* gene. The *deaD* gene codes for a polypeptide of 624 amino acids.

#### [0077] Example 3

Preparation of the expression vector pXK99E*deaD* for IPTG-induced expression of the *deaD* gene in *C. glutamicum*

#### [0078] 3.1 Cloning of the *deaD* gene

From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)) *I.B.R.* On the basis of the sequence of the *deaD* gene known for *C. glutamicum* from example 2, the following oligonucleotides were chosen for the polymerase chain reaction (see SEQ ID No. 3 and SEQ ID No. 4):

*deaD* for2:

5`-GA TCT AGA-AAT CCG GCT TCG ATG CAC GC-3` SEQ ID NO: 3

dead int2:

5`- CT AAG CTT-CGA CGG TTG GCA GTT CCA TT-3` SEQ ID NO: 4

[0079] The primers were chosen here so that the amplified fragment contains the incomplete gene, starting with the native ribosome binding site without the promoter region, and the front region of the dead gene. Furthermore, the primer dead for2 contains the sequence for the cleavage site of the restriction endonuclease XbaI, and the primer dead int2 the cleavage site of the restriction endonuclease HindIII, which are marked by underlining in the nucleotide sequence shown above.

[0080] The primers shown were synthesized by MWG-Biotech AG (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) I.B.R. with Pwo-Polymerase from Roche Diagnostics GmbH (Mannheim, Germany). With the aid of the polymerase chain reaction, the primers allow amplification of a DNA fragment 1132 bp in size, which carries the incomplete dead gene, including the native ribosome binding site.

[0081] The dead fragment 1132 bp in size was cleaved with the restriction endonucleases XbaI and HindIII and then isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

[0082] 3.2 Construction of the expression vector pXK99E

The IPTG-inducible expression vector pXK99E was constructed according to the prior art. The vector is based on the Escherichia coli expression vector pTRC99A (Amann et al., Gene 69: 301-315 (1988) I.B.R.) and contains the trc promoter, which can be induced by addition of the lactose derivative IPTG (isopropyl  $\beta$ -D-thiogalactopyranoside), the termination regions T1 and T2, the replication origin ColE1 from E. Coli, the lacI<sup>q</sup> gene (repressor of the lac operon



from E.coli), a multiple cloning site (mcs) (Norrande, J.M. et al. Gene 26, 101-106 (1983) I.B.R.) and the kanamycin resistance gene aph(3')-IIa from E. coli (Beck et al. (1982), Gene 19: 327-336 I.B.R.).

[0083] It has been found that the vector pXK99E is quite specifically suitable for regulating the expression of a gene, in particular effecting attenuated expression in coryneform bacteria. The vector pXK99E is an E. coli expression vector and can be employed in E. coli for enhanced expression of a gene.

[0084] Since the vector cannot replicate independently in coryneform bacteria, this is retained in the cell only if it is integrated into the chromosome. The peculiarity of this vector here is the use for regulated expression of a gene after cloning of a gene section from the front region of the corresponding gene in the vector containing the start codon and the native ribosome binding site, and subsequent integration of the vector into coryneform bacteria, in particular C. glutamicum. Gene expression is regulated by addition of metered amounts of IPTG to the nutrient medium. Amounts of 0.5  $\mu$ M/l up to 10  $\mu$ M/l IPTG have the effect of very weak expression of the corresponding gene, and amounts of 10  $\mu$ M/l up to 100  $\mu$ M/l have the effect of a slightly attenuated to normal expression of the corresponding gene.

[0085] The E. coli expression vector pXK99E constructed was transferred by means of electroporation (Tauch et al. 1994, FEMS Microbiol Letters, 123: 343-347 I.B.R.) into E. coli DH5 $\alpha$ mcr (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649 I.B.R.). Selection of the transformants was carried out on LB Agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989 I.B.R.), which had been supplemented with 50 mg/l kanamycin.

[0086] Plasmid DNA was isolated from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 - 927 *I.B.R.*), cleaved with the restriction endonuclease NcoI, and the plasmid was checked by subsequent agarose gel electrophoresis.

[0087] The plasmid construct obtained in this way was called pXK99E (figure 1). The strain obtained by electroporation of the plasmid pXK99E in the *E. coli* strain DH5 $\alpha$ mcr was called *E. coli* DH5 $\alpha$ lphamcr/pXK99E (= DH5 $\alpha$ mcr/pXK99E) and deposited on 31st July 2001 as DSM 14440 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

[0088] 3.3 Cloning of the *deaD* fragment in the *E. coli* expression vector pXK99E

The *E. coli* expression vector pXK99E described in example 3.2 was used as the vector. DNA of this plasmid was cleaved completely with the restriction enzymes XbaI and HindIII and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250).

[0089] The *deaD* fragment approx. 1120 bp in size described in example 3.1, obtained by means of PCR and cleaved with the restriction endonucleases XbaI and HindIII was mixed with the prepared vector pXK99E and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation batch was transformed in the *E. coli* strain DH5 $\alpha$ mcr (Hanahan, In: DNA cloning. A Practical Approach. Vol. I, IRL-Press, Oxford, Washington DC, USA *I.B.R.*). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190 *I.B.R.*) with 50 mg/l kanamycin. After

incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and cleaved with the restriction enzymes XbaI and HindIII to check the plasmid by subsequent agarose gel electrophoresis. The resulting plasmid was called pXK99EdeaD. It is shown in figure 2.

#### [0090] Example 4

Integration of the vector pXK99EdeaD into the genome of the *C. glutamicum* strain DSM5715

[0091] The vector pXK99EdeaD mentioned in example 3 was electroporated by the electroporation method of Tauch et al., (1989 FEMS Microbiology Letters 123: 343-347) *I.B.R.* in the strain *C. glutamicum* DSM5715. The vector cannot replicate independently in DSM5715 and is retained in the cell only if it has integrated into the chromosome. Selection of clones with integrated pXK99EdeaD was carried out by plating out the electroporation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989 *I.B.R.*), which had been supplemented with 15 mg/l kanamycin and IPTG (1mM/l).

[0092] For detection of the integration, the *deaD* fragment was labeled with the Dig hybridization kit from Boehringer by the method of "The DIG System Users Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993 *I.B.R.*). Chromosomal DNA of a potential integrant was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)) *I.B.R.* and in each case cleaved with the restriction enzymes BstEII and XbaI. The fragments formed were separated by means of agarose gel electrophoresis and hybridized at 68°C with the Dig hybridization kit from Boehringer. The plasmid pXK99EdeaD

mentioned in example 3 had been inserted into the chromosome of DSM5715 within the chromosomal *deaD* gene. The strain was called DSM5715::pXK99E*deaD*.

[0093] Example 5

Preparation of lysine

[0094] The *C. glutamicum* strain DSM5715::pXK99E*deaD* obtained in example 4 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined. By addition of IPTG, attenuated expression of the *deaD* gene occurs, regulated by the *trc* promoter.

[0095] For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l) and IPTG (10  $\mu$ M/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium Cg III was used as the medium for the preculture.

| [0096]                          | Medium Cg III |
|---------------------------------|---------------|
| NaCl                            | 2.5 g/l       |
| Bacto-Peptide                   | 10 g/l        |
| Bacto-Yeast extract             | 10 g/l        |
| Glucose (autoclaved separately) | 2% (w/v)      |

The pH was brought to pH 7.4

Kanamycin (25 mg/l) and IPTG (10  $\mu$ M/l) were added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. The OD (660 nm) of the preculture was 0.8. 450  $\mu$ l of this preculture were transinoculated into a main culture such that the initial OD (660 nm) of the main culture was 0.1. By transfer of IPTG-containing

medium from the preculture, the IPTG concentration in the main culture was approx. 0.5  $\mu$ M/l. Medium MM was used for the main culture.

**[0097]**

Medium MM

|   |          |
|---|----------|
| CSL (corn steep liquor)                         | 5 g/l    |
| MOPS (morpholinopropanesulfonic acid)           | 20 g/l   |
| Glucose (autoclaved separately)                 | 50g/l    |
| Salts:  |          |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 25 g/l   |
| KH <sub>2</sub> PO <sub>4</sub>                 | 0.1 g/l  |
| MgSO <sub>4</sub> * 7 H <sub>2</sub> O          | 1.0 g/l  |
| CaCl <sub>2</sub> * 2 H <sub>2</sub> O          | 10 mg/l  |
| FeSO <sub>4</sub> * 7 H <sub>2</sub> O          | 10 mg/l  |
| MnSO <sub>4</sub> * H <sub>2</sub> O            | 5.0mg/l  |
| Biotin (sterile-filtered)                       | 0.3 mg/l |
| Thiamine * HCl (sterile-filtered)               | 0.2 mg/l |
| Leucine (sterile-filtered)                      | 0.1 g/l  |
| CaCO <sub>3</sub>                               | 25 g/l   |

The CSL, MOPS and the salt solution are brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions are then added, and the CaCO<sub>3</sub> autoclaved in the dry state is added.

**[0098]** Culturing was carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) was

added. Culturing was carried out at 33°C and 80% atmospheric humidity.

[0099] After 67 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

[0100] The result of the experiment is shown in table 1.

Table 1

| Strain              | OD<br>(660 nm) | Lysine HCl<br>g/l |
|---------------------|----------------|-------------------|
| DSM5715             | 7.6            | 13.57             |
| DSM5715::pXK99EdeaD | 12.2           | 16.31             |

[0101] This application claims priority to German Priority Document Application No. 100 47 865.4, filed on September 27, 2000. The above German Priority Document is hereby incorporated by reference in its entirety.